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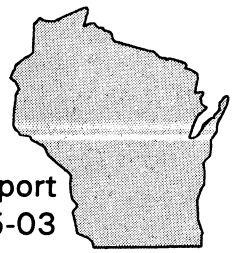

Groundwater Research Report
WRC GRR 95-03

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This project was supported, in part, by General Purpose Revenue funds of the State of Wisconsin to the University of Wisconsin System for the performance of research on groundwater quality and quantity. Selection of projects was conducted on a competitive basis through a joint solicitation from the University and the Wisconsin Departments of Natural Resources; Agriculture, Trade and Consumer Protection; Labor and Human Relations; and with the advice of the Wisconsin Groundwater Research Advisory Council and the concurrence of the Wisconsin Groundwater Coordinating Council.

ABSTRACT

The contamination of soils, sediments, groundwater and surface waters by herbicides poses major cleanup problems. This project focuses on atrazine, but involves development of a bioremediation protocol applicable for herbicides. Atrazine is one of the most commonly detected organic contaminants in groundwater throughout Wisconsin. The report describes initial experiments evaluating a conceptual computer model used to establish experimental methodology and design for selective enrichment of aerobic mixed microbial cultures capable of accelerated destruction of atrazine. Preliminary studies have focused on development of analytical procedures to determine the concentration of atrazine and its metabolites, ammonium, and microbial biomass. Values derived from these experiments have been used to determine initial input parameters for the computer model using *Pseudomonas* sp. strain D. Recoveries of atrazine by gas chromatography were variable (74 to 123%). Recoveries of cyanuric acid using high pressure liquid chromatography (HPLC) were 100.0 ± 0.3%. HPLC was the method of choice to detect atrazine metabolites. *Pseudomonas* sp. strain D, under ultimately nitrogen-limiting conditions, had an average specific growth rate of 0.27 ± 0.02 hr^{-1}, a 44.6 ± 1.5% protein content, and a specific growth yield of 45.8 ± 1.5 grams of protein produced per mole of nitrogen consumed. *Pseudomonas* sp. strain D, under ultimately carbon-limiting conditions, had an average specific growth rate of 0.34 ± 0.02 hr^{-1}, a 45.5 ± 4.3% protein content, and specific growth yield of 40.6 ± 13.5 grams of protein produced per mole of nitrogen consumed. Values from both growth experiments were explained by the model, thereby verifying the model's usefulness.

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INTRODUCTION

The contamination of soils, sediments, groundwater and surface waters by herbicides is a major environmental problem. Bioremediation methods of *in situ* treatment offer alternative and/or supplementary approaches to the costly landfill or incinerator methods of disposal. The project focuses specifically on atrazine, but is applicable to other herbicides.

Atrazine is the most commonly detected organic contaminant in groundwaters of the Midwest (Hallberg, 1989). In Wisconsin, atrazine detection levels and frequencies in groundwater wells vary considerably. For example, in a survey of Grade A dairy farms, atrazine was detected in 12% of the wells, while 7% of these wells had concentrations exceeding the preventive action limit (PAL) of 0.35 ppb (LeMasters and Doyle, 1989). In contrast, atrazine was detected in 71% of the wells from high-risk areas with concentrations exceeding the PAL in 51%.

Atrazine contaminates groundwater by leaching through the soil at sites where it is mixed, improperly disposed of, or applied to fields for weed control. The leaching potential is increased by the relative persistence of the chemical in soil with half-lives ranging from 1.5 to 6 months (Goring et al., 1975). Chemical hydrolysis is slow under normal soil pH conditions (Armstrong et al., 1967). Microbiological degradation of atrazine in soil is limited in extent (e.g., $<10\%$ of the ethyl side chain), occurs at relatively slow rates and is a function of soil temperature, moisture, and organic carbon content (McCormick and Hiltbold, 1966; Roeth et al., 1969; Goswami and Green, 1971; Skipper and Volk, 1972; Geller, 1980). Degradation pathways of atrazine by microorganisms have been elucidated in pure cultures and are illustrated in Figure 1 (Cook and Hutter, 1981; Giardinia et al., 1982; Behki and Khan, 1986; Cook, 1987; Erickson and Lee, 1989).

Atrazine has been shown to support growth of microorganisms (Cook, 1987; Erickson and Lee, 1989). The bioenergetic incentive for microbial degradation of atrazine and its metabolites is found in the alkyl side chains since the ring carbon atoms are at the same oxidation state as CO_2. Degradation of desalkylated atrazine metabolites occurs when they are utilized by microorganisms as nitrogen sources (Cook, 1987). Microbial growth yields based on reductance degree--a measure of the available electrons associated with growth substrates--and related energy potential (Harris, 1982; Esener et al., 1983) of *s*-triazines are the most appropriate yield indices for evaluating *s*-triazine degradation (Erickson and Lee, 1989). Interpretation of existing, limited growth yields on *s*-triazines is complicated by low substrate solubility-related phenomena and ill-defined cultural conditions (Cook, 1987).

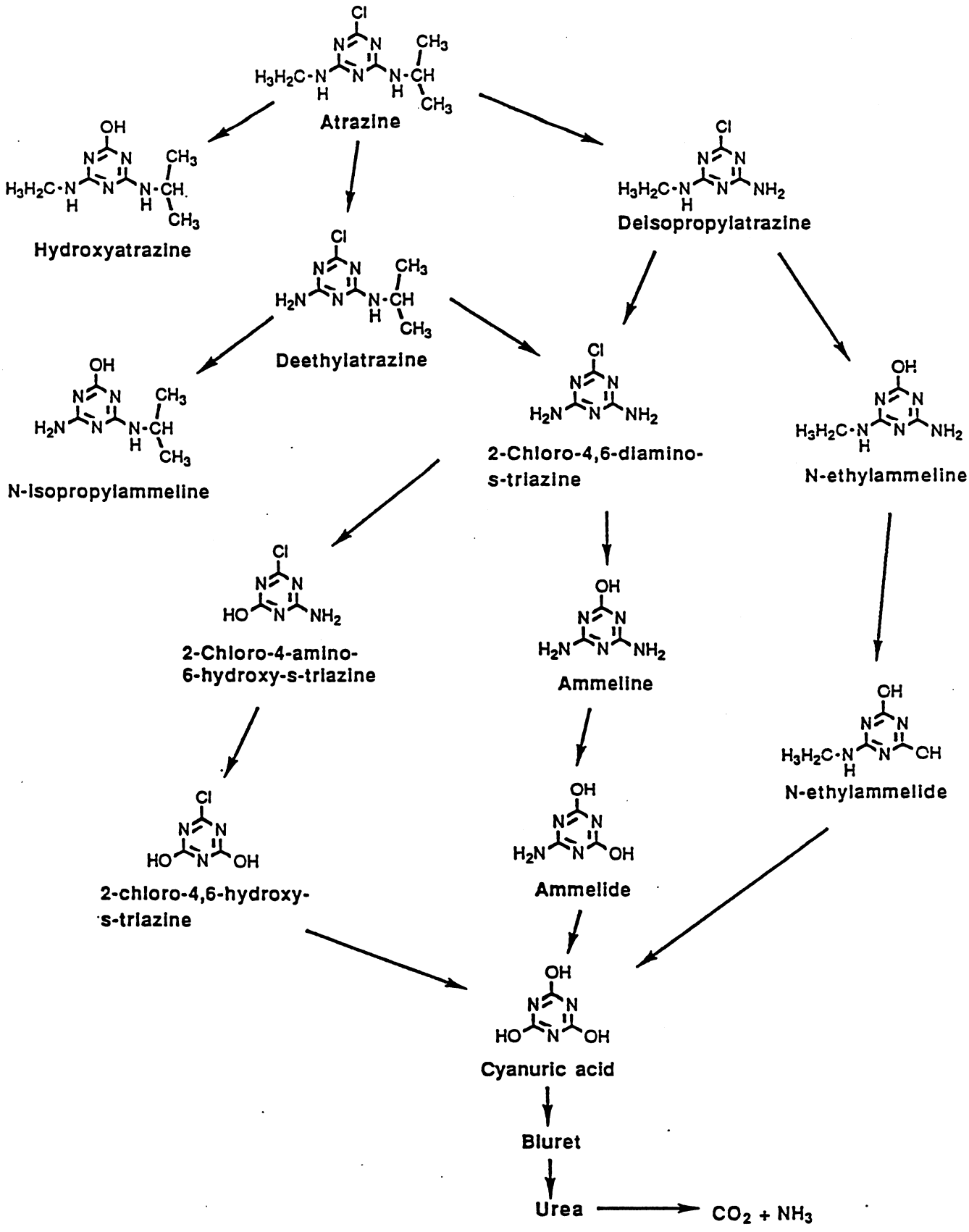


Figure 1. Degradation pathways of atrazine by micorbial pure cultures.

Information on the anaerobic nitrate reduction and denitrification performed by *Pseudomonas* sp. strain D does not exist. The results of the experiments aid in describing conditions for microbial degradation of atrazine in groundwater and in other systems with low dissolved oxygen. In addition to serving as models of microbial system performance, the results could aid in predicting the fate of atrazine in groundwater.

This report describes initial experiments evaluating a conceptual computer model to facilitate experimental design for selective enrichment of aerobic mixed microbial cultures capable of effecting accelerated destruction of atrazine and its metabolites. The models provide a basis for understanding, interpreting, predicting, and controlling the performance of bioremediation systems. In addition, they facilitate experimental design for selective enrichment of mixed microbial cultures capable of effecting accelerated destruction of xenobiotics such as atrazine by pathways that bypass undesirable intermediates. These preliminary studies have focused on analytical procedures to determine concentrations of atrazine and its metabolites, ammonium, and microbial biomass. These values have been used to determine initial input parameters for the model using strain D, an established triazine-degrader. Experiments are also described investigating the ability of strain D and *Klebsiella pneumoniae* (another established triazine degrader) to utilize nitrate as an assimilatory nitrogen source and dissimulatory electron acceptor.

MATERIALS AND METHODS

BACTERIAL CULTURES

Strain D and *K. pneumoniae* were obtained from CIBA-GEIGY (Greensboro, North Carolina). The relevant phenotypes of these organisms are summarized in Table 1. Stock cultures of these organisms were maintained on agar plates and slants containing cyanuric acid-glucose (CAG) media described below. *Pseudomonas aeruginosa* and *Escherichia coli* were maintained on agar plates and slants containing Luria-Bertani (LB) media described below. All cultures were stored at 4°C and transferred periodically to ensure their purity.

Table 1. Summary of atrazine metabolite mineralization.

Organism	Metabolite Utilized
Pseudomonas sp. strain D	N-isopropylammelide Nethylammelide Ammeline Ammelide Cyanuric Acid
K. pneumoniae strain 99	Ammelide Cyanuric Acid

GROWTH MEDIA

Cells were grown in CAG media (modified from Cook and Hutter, 1981), which consisted of 10 m*M* potassium phosphate pH 7.3 buffer, 0.25 *M* $MgSO_4$, 5 m*M* glucose as the carbon source, cyanuric acid (2.5 m*M* N) and trace elements (Pfennig and Lippert, 1966). Carbon and nitrogen sources were sterilized by membrane filtration using a 0.22 μm pore diameter filter. An appropriate volume of this media was transferred aseptically to a 250 mL sidearm Erlenmeyer flask just before use. LB medium consisted of (per liter) 10 g tryptone, 5 g yeast extract, and 9.5 g NaCl.

COMPUTER MODELING

The models use concepts and equations detailed in Harris (1982), Andrews and Harris (1986), and Cascino et al. (1990), and the framework of a first-approximation model simulating metolachlor mineralization by a microbial consortium in continuous culture (Khan et al., 1988). Structurally, the models are a set of spreadsheets defining

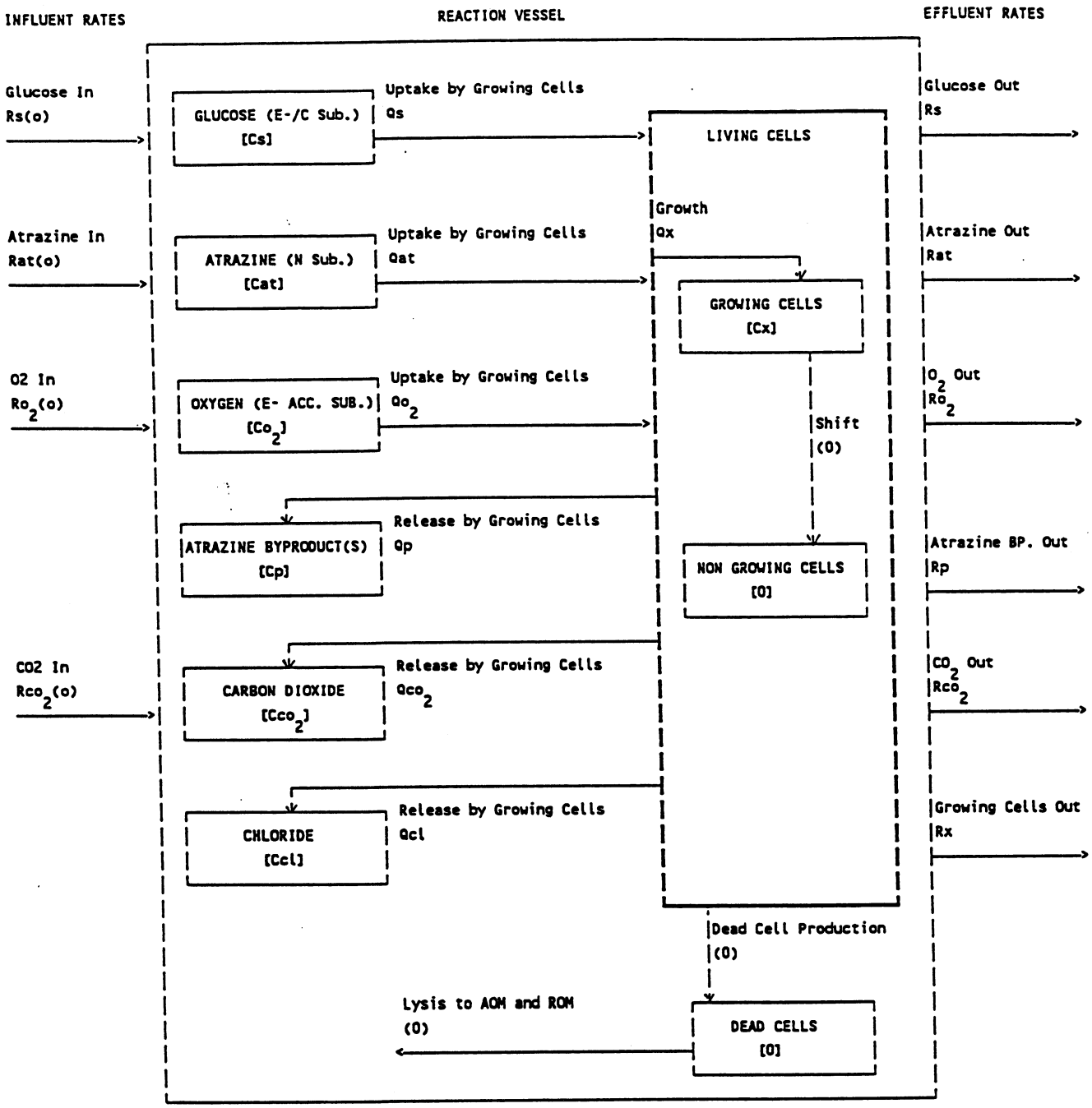
ecophysiological properties, interlinked with spreadsheets defining system conditions and data output. The ecophysiological spreadsheets are interlinked, and define (1) cell physical composition, (2) cell polymer and chemical composition, (3) cell and metabolite elemental composition, reluctance degree and energy potential composition, (4) boundary specific rate and efficiency of substrate uptake and cell growth, maintenance, death and lysis coefficients, and (5) stoichiometric mass balance coefficients for substrate uptake and product release. The conceptual structure of the model is illustrated schematically in Figure 2. The structure of the data output spreadsheets depends on the experimental goal. Current models emphasize fed-batch and batch cultures limited by a preferred organic substrate, with atrazine as non-limiting target substrate used cometabolically or non-cometabolically by the microbial population only under conditions of limiting preferred substrate concentration. The models describe the mass balance kinetics of system variables and data output properties such as media flow rate and input and output concentrations of substrates and products (including preferred and target organic substrate and intermediary degradation products, microbial biomass, ammonium, oxygen, carbon dioxide, pH, and chloride).

GAS CHROMATOGRAPHY

A Hewlett-Packard 5890A gas chromatograph (GC) fitted with a Hewlett-Packard 7673A auto injector, a Hewlett-Packard 3393A integrator, and a nitrogen-phosphorus detector was used to determine the concentration of atrazine in cultures. Samples (1 μl) were injected on a Hewlett-Packard 17 fused silica capillary megabore (10 m x 0.53 mm) column, containing a 50% phenyl 50% methylpolysiloxane matrix of intermediate polarity. Helium was used as the carrier gas at a flow rate of 10 mL/min. The column temperature was increased from 180 to 240°C at a rate of 4°C/min. The injector and detector temperatures were maintained at 225 and 260°C, respectively.

HIGH-PRESSURE LIQUID CHROMATOGRAPHY

High-pressure liquid chromatography (HPLC) was used to determine the concentrations of *s*-triazines in culture. Two HPLC methods were used. Both utilized a Gilson HPLC system equipped with model 302 pumps and a model 116 dual wavelength ultraviolet (UV) detector (220/240/nm). The first method utilized an Alltech Mixed Mode Cation Exchange Reverse Phase (RP)-C8 column. An isocratic mobile phase consisting of 68% phosphate buffer (pH 6.7) and 32% methanol with a flow rate of 0.89 mL/min. The second method (Beilstein et al., 1981) employed a 25 cm Supelco ODS column with a C-18 RP stationary phase (5 μm mean particle diameter). Separation involved a mobile phase gradient that varied from 100% 100 m*M* phosphate buffer (pH 6.7) to 70% methanol in 10 m*M* phosphate buffer (pH 6.7) at a flow of 0.5 mL/min. Sample (20 μl) was injected and after 10 min, the linear gradient was started. Sample elution was complete in 50 min, at which time the gradient was reversed. Starting conditions were reestablished within 60 min of initial



KEY

Subscript w: w = substrate (e.g., e-/C substrate, s), cells (x) or metabolic product (e.g., p); Cw: concentration of substrate, cell or metabolic product (mole w/L); Rw(o): rate of input of metabolite into the system (mole w/L°h); Rw: rate of output of metabolite from system (mole w/L°h); Qw: rate of substrate consumption, cell production or metabolic product production (mole w/L°h); I: specific growth rate of cells (1/h); AOM: available organic matter; and ROM: recalcitrant organic matter.

Figure 2. Pure culture aerobic heterotroph growing on glucose (E-/C source) and atrazine (N source): Description of mass balance cell and metabolite pools and processes.

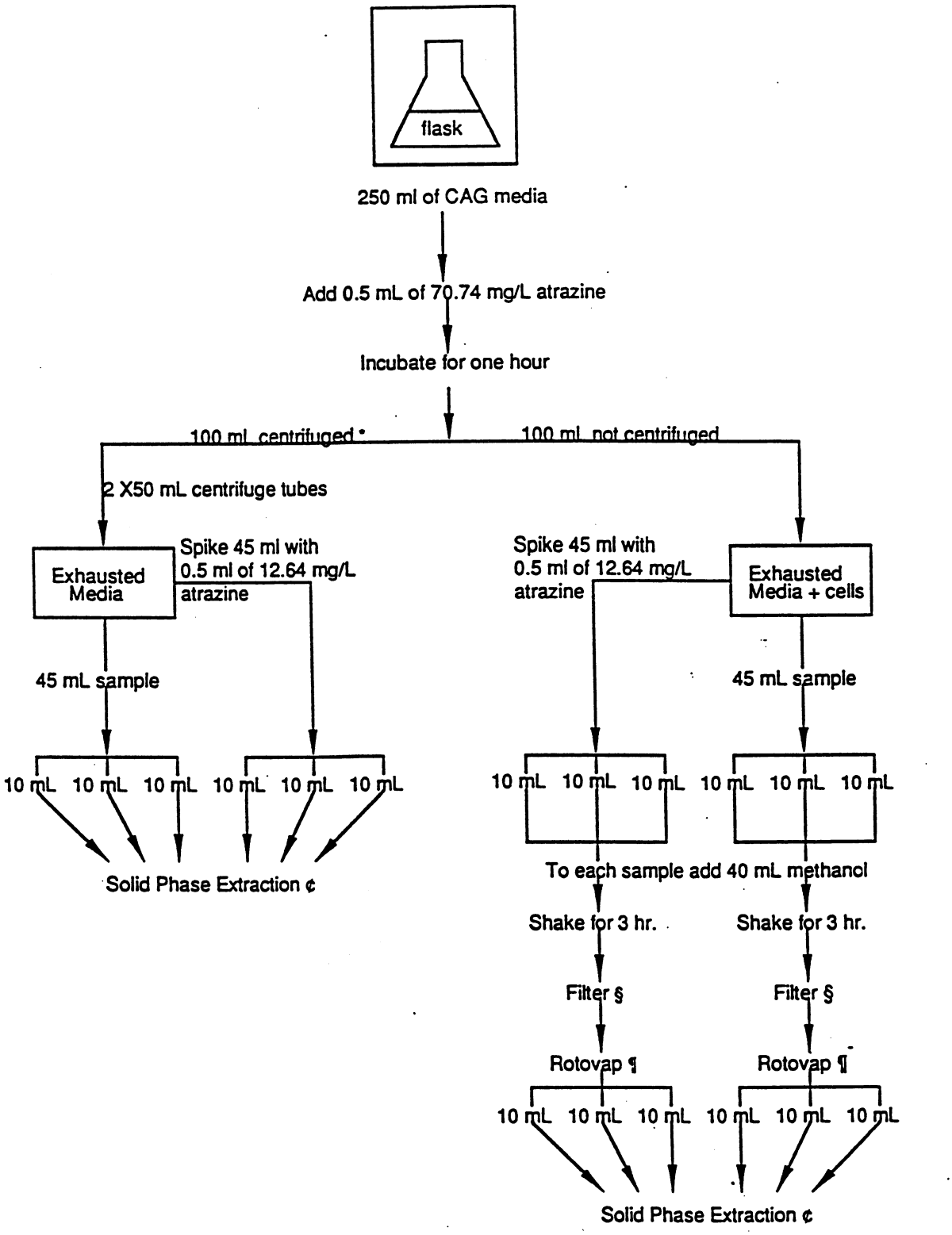
injection. Samples from cell cultures were prepared by centrifugation (15,000 rpm, 30 min, 4°C). The supernatant (3 mL) was transferred to a vial and 20 μl was injected directly onto the HPLC column. Samples were quantified against a standard curve.

RECOVERY OF ATRAZINE FROM CELL CULTURE

The extraction procedure (Figure 3) consists of: soil (1 g) in a flask containing 250 mL of CAG media was incubated at 21°C with rotary shaking at 200 rpm for 2 days, and 1 mL was transferred to 250 mL of CAG media. After 2 days of incubation, 0.5 mL of atrazine (70.75 mg/liter in methanol) was added to the cell suspension and incubated for 1 hr. Two 100-mL samples were collected, one was centrifuged, the other was not. Both 100 mL samples were divided into 45 mL subsamples, and one 45-mL subsample from each set received a second spike of 0.5 mL 12.64 mg/liter atrazine. Each 45-mL subsample was divided into 10-mL aliquots. The centrifuged aliquots were extracted by solid phase extraction (SPE) using 6 mL 0.5 g Superclean Envi-18 solid phase extraction tubes installed on a vacuum manifold. The twelve 10 mL aliquots were run through the SPE tubes at a rate of 3 mL/min. After the sample had dripped through, the columns were dried for 15 min with a gentle stream of nitrogen. Atrazine was eluted from the columns by pulling three 1-mL aliquots of methanol through at a rate of 1 mL/min into 10-mL volumetric flasks. The flasks were filled to volume with methanol and two 3-mL replicates of each of the 12 samples were transferred to GC vials for analysis. 40 mL of methanol was added to each of the non-centrifuged 10-mL aliquots. They were shaken for 3 hr, suction filtered using a 47-mm, 45-μm millipore filter, and rotovapped to drive off the methanol. These samples were ready for solid phase extraction.

RECOVERY OF CYANURIC ACID FROM CELL CULTURE

Known amounts of cyanuric acid were added to 50 mL cell cultures, the analyte concentrations were quantified against a standard curve of concentrations ranging from 0.709 to 7.085 mg/liter. The HPLC method utilized a mixed mode cation exchange column. Cells were grown in the mineral salts medium described above containing 5 m*M* glucose and 2.5 m*M* ammonium, and 100 mL of media was inoculated with 1g of soil and incubated on a rotary shaker at 200 rpm for 35 hr at 21 ± 2°C. Fresh medium (49 mL) was inoculated with 1 mL of soil suspension and incubated for 2 days. After 2 days, 1 mL of 236.18 mg/liter cyanuric acid was spiked into the flask and allowed to shake for 1 hr. After 1 hr, three 10-mL samples were collected and centrifuged (9,000 rpm, 20 min, 4°C). Supernatant (2.7 mL) was collected and added to 0.3 mL of 1 *M* HCl and frozen until analyzed. On thawing, 0.3 mL of 1 *M* NaOH was added to the sample to give a final cyanuric acid concentration of 3.789 ppm which was injected directly (20 μL) onto the HPLC column.



KEY

*3000 rpm for 1 hour; §Millipore type HA 0.45 μm filters; ¶Büchi Rotovaper R110, RE111 55-60°C @ 60-70 cB; ¢Supelco Superclean™ Envi-18™ SPE tubes 6 ml, 0.5 g.

Figure 3. Recovery of atrazine from cell culture.

AMMONIUM DETERMINATION

Ammonium was determined by a Kjeldahl steam distillation technique (modified from Bremner and Edwards, 1965). Acidified samples collected previously were brought to room temperature and 1 mL of sample was added to 9 mL of distilled water and 0.5 mL of 1 *N* NaOH. Samples were distilled for 3 min and titrated to pH 5.1 with 0.000992 *N* NaOH. Standards containing 1 mL of 1 μM $(NH_4)_2SO_4$, 9 mL distilled water and 0.5 mL 1 *N* NaOH were distilled to calibrate the acid.

BIOMASS BY TURBIDITY MEASUREMENTS

Turbidity measurements in batch culture were determined by periodic measurements on sidearm erlenmeyer flasks using a Klett-Sommerson colorimeter with a blue #42 filter. The growth rate of the culture was determined by plotting the natural log of growth vs. time and computing the slope of the linear portion of the curve.

BIOMASS BY DRY WEIGHT DETERMINATIONS

Dry weight measurements were performed by membrane filtration (Stouthamer, 1969) using duplicate 15 to 25 mL aliquot samples. Millipore membrane filters of 0.45 μm pore size, 47 mm diameter, were soaked in deionized water and washed with 3 volumes of 10 mL deionized water by suction-filtration using a vacuum pump set at 20 psi. The filters were dried to constant weight at 105°C for 2 hr, cooled in a vacuum desiccator, weighed, and stored in a desiccator. The sample aliquot to be measured was centrifuged at 10,000 rpm (12,062 *g*) for 10 min in a 50-mL polypropylene centrifuge tube at 15 to 20°C. The supernatant was filtered through the prewashed, preweighed membrane filter into a 50-mL Pyrex culture tube. When filtration was complete the 50-mL discard tube was replaced by 50-mL collection tube. The pellet was resuspended in a minimal volume of deionized water, transferred to the filter and dewatered. The cells and filter paper were dried to constant weight at 105°C for 2 hr and cooled in a vacuum desiccator prior to weighing.

BIOMASS BY PROTEIN DETERMINATION

Protein was determined by the method of Bradford (1976). Samples (3 mL) from growing cell suspensions were mixed with an equal volume of 1.0 *M* NaOH, boiled for 10 min, and stored at 4°C for further analysis. The solution was allowed to reach room temperature, and clarified by centrifugation (3,200 rpm, 5 min in microcentrifuge tubes). Supernatant was collected (1 mL) and 0.5 mL of 0.1 *M* HCl was added to neutralize the base. Two volumes of protein reagent (Coomassie Brilliant Blue G-250 in 95% ethanol and 85% [w/v] phosphoric acid) were added to the 1.5-mL sample or the 1.5-mL bovine serum albumin standards (2.20 to 60.60 μg/mL). The samples and standards were incubated at room temperature for 15 min before absorbance readings were taken at 595 nm on a Perkin-Elmer Lambda UV/VIS

spectrophotometer. The instrument was blank corrected with a solution containing 1.5 mL of 0.15 *M* NaCl and 3 mL of protein reagent.

BATCH CULTURE CHARACTERIZATION OF *PSEUDOMONAS* SP. STRAIN D

Growth of strain D was monitored by measuring turbidity, dry weight, and protein. For all growth experiments, a 250 mL side-arm erlenmeyer flask containing the appropriate volume of media was aseptically inoculated from the stock culture. Flasks were incubated in a constant temperature room (21 ± 2°C) with rotary shaking (200 rpm) until the limiting nutrient was exhausted, and growth had ceased. This was ascertained when two successive turbidity measurements were essentially identical. A 1% (e.g., 0.5 to 0.75 mL) inoculum was added to a 250-mL side-arm erlenmeyer flask containing the same medium used for the starter culture. Growth yields were calculated by determining the amount of biomass produced (measured as protein or dry weight) when the limiting nutrient was exhausted. Percent protein was determined by measuring the amount of protein and cell material produced. The percent nitrogen was determined by measuring the amount of nitrogen consumed and the amount of biomass produced.

NITRATE REDUCTION AND DENITRIFICATION

Nitrate reduction and denitrification by strain D and *K. pneumoniae* were investigated. *P. aeruginosa* (a known denitrifier), and *E. coli* (reduces nitrate to nitrite) were used as positive controls. Non-inoculated medium and medium lacking nitrate but inoculated with *P. aeruginosa* were used as negative controls. Cell suspensions used as inocula were precultured in LB medium. The assays were conducted in 20-mL screw top test tubes containing 10 mL of denitrification medium (LB supplemented with 0.1% KNO_3 or KNO_2 and 0.17% agar). The media were inoculated with the appropriate organism by gently mixing the inoculum with the medium to distribute it throughout the tube. To detect the ability of the organisms to reduce nitrate to nitrite a 0.1-mL sample from test tubes was added to 2 mL of test reagent (N-(1-Naphthyl)-ethylenediamine dihydrochloride and sulfanilic acid), (Smibert and Krieg, 1981). Water was added for a final volume of 4 mL and the color was allowed to develop for 15 min. A red color indicated the presence of nitrite. If red color failed to develop, approximately 5 mg of granular zinc was added to the solution, which would result in formation of red color by the chemical reduction of nitrate to nitrite. If red color still failed to appear, then complete denitrification was indicated. The assay for strain D was carried out for 9 days and samples were analyzed every third day. For *K. pneumoniae*, the assay was carried out for 6 days and samples were analyzed every third day. Negative and positive controls were sampled concurrently with the two test organisms.

RESULTS AND DISCUSSION

A critical measurement necessary for the model is the concentration of atrazine in the cultures. To this end, experiments were conducted to determine the efficiency by which the SPE procedure could recover atrazine from aqueous cultures. Recovery of atrazine from 24 aqueous cell cultures was highly variable, ranging from 74 to 123% (Table 2). Variability in recoveries was further complicated by the low degree of precision associated with replicates and standards analyzed by GC (Table 2). In this respect, results between sample replicates were at best ± 3%. Thus, the high variability of the percent recoveries of atrazine indicates problems with the GC and not the extraction procedure, but further investigations are required to determine this. The low degree of precision by the GC indicates the need for an internal standard.

The determination of cyanuric acid from cell cultures gave recoveries of 100.0 ± 0.3% for three samples (prepared in duplicate). The standard curve of cyanuric acid gave an R^2 value of 0.9995 (Table 3). Cyanuric acid had a retention time of approximately 2.7 min using the mixed mode cation exchange method. This was very close to the elution time of the water peak, indicating the compound was not retained on the column (Figure 4). Thus, although recoveries of cyanuric acid were excellent, the utility of the method for determining this compound in complex mixtures may be limited. With the second HPLC method (Beilstein et al., 1981), cyanuric acid had a retention time of approximately 8.5 min indicating retention on the column (Figure 5). The second HPLC method is useful for determination of atrazine metabolites. Recent investigations using this method showed very good separation of seven other atrazine metabolites and the parent compound (Figure 6). While good resolution was achieved, a very long run time was required (60 min).

Three separate experiments were conducted to determine the accuracy of the biomass and ammonium determinations and to determine the growth characteristics of strain D (Table 4). In the first experiment, ammonium and cyanuric acid were limiting nitrogen sources and turbidity was monitored as a function of growth at 30°C. Strain D readily used ammonium and cyanuric acid as limiting nitrogen sources with specific growth rates of 0.33 ± 0.01 hr^{-1} and 0.30 ± 0.03 hr^{-1}, respectively. Strain D showed a longer lag phase when utilizing cyanuric acid (Figure 7).

In the second experiment, ammonium was the limiting nitrogen source and turbidity, protein, dry weight, and ammonium were monitored. Strain D had an average specific growth rate of 0.27 ± 0.02 hr^{-1} (Figure 8). It had a 44.6 ± 1.5% protein content, 15.2 ± 1.3% nitrogen content, and an average growth yield of 45.8 ± 1.5 g protein produced/nitrogen mole consumed. The percent nitrogen probably is an overestimation of the true value because samples (15 mL) collected for the dry weight determinations were of insufficient volume to make an accurate determination.

Table 2. Recovery of atrazine from cell culture.

Sample Numer	Sample Medium	Conc. of 1st spike (μg/ml)	Conc. of 2nd spike (μg/ml)	Area (GC)	Calculated concentration GC (μg/ml)	Calculated concentration "Real" (μg/ml)	Recovery (%)
A 1-1-nsp	CAG-cells	0.1412	0	9326	0.13	0.141	89.81
A 1-1-nsp	CAG-cells	0.01412	0	8127	0.1106	0.141	78.29
A 2-1-nsp	CAG-cells	0.1312	0	9991	0.1358	0.141	96.2
A 2-2-nsp	CAG-cells	0.1412	0	7690	0.1046	0.141	74.09
A 3-1-nsp	CAG-cells	0.1412	0	8844	0.1203	0.141	85.18
A 3-2-nsp	CAG-cells	0.1412	0	9805	0.1333	0.141	94.41
B 1-1-sp	CAG+cells	0.1412	0.1389	20443	0.2776	0.28	99.12
B 1-2-sp	CAG+cells	0.1412	0.1389	17521	0.238	0.28	84.97
B 1-2-sp	CAG+cells	0.1412	0.1389	21471	0.2916	0.28	104.1
B 2-2-sp	CAG+cells	0.1412	0.1389	19178	0.2604	0.28	93
B 3-1-sp	CAG+cells	0.1412	0.1389	21191	0.2878	0.28	102.75
B 3-2-sp	CAG+cells	0.1412	0.1389	19837	0.2694	0.28	96.19
C 1-1-nsp	CAG-cells	0.1412	0	10537	0.1432	0.141	101.44
C 1-2-nsp	CAG-cells	0.1412	0	11338	0.1541	0.141	109.13
C 2-1-nsp	CAG-cells	0.1412	0	10416	0.1416	0.141	100.28
C 2-2-nsp	CAG-cells	0.1412	0	10799	0.1468	0.141	103.96
C 3-1-nsp	CAG-cells	0.1412	0	10617	0.1443	0.141	102.21
C 3-2-nsp	CAG-cells	0.1412	0	10166	0.1382	0.141	97.88
D 1-1-nsp	CAG+cells	0.1412	0.1389	23972	0.3255	0.28	116.21
D 1-2-nsp	CAG+cells	0.1412	0.1389	22980	0.312	0.28	111.41
D 2-1-nsp	CAG+cells	0.1412	0.1389	23827	0.3235	0.28	115.51
D 2-2-nsp	CAG+cells	0.1412	0.1389	23758	0.3226	0.28	115.18
D 3-1-nsp	CAG+cells	0.1412	0.1389	25471	0.3458	0.28	123.47
D 3-2-nsp	CAG+cells	0.1412	0.1389	25424	0.3452	0.28	123.25

†Sample Description: A- sample .....; D- ... SPE.
Sample Volumes: Primary - 10 ml; Secondary - 0 ml; and Final - 5 ml
GC volume with successive sample injections - 2 μL.

Linear Regression of Atrazine Standards

Standard Number	Concentration (μg/ml)	Retention (min)	Area (GC)	Estimated Concentration
I	0.051	5.455	1697	0.0467
II	0.126	5.405	4859	0.1325
III	0.354	5.421	13151	0.3574
IV	0.632	5.447	22929	0.6227
V	1.264	5.426	46666	1.2666

Regression Output:

Constant	-24.0859
Std Err of Y Est	267.0846
R-squared	0.999836
No. of Observations	5
Degrees of Freedom	3
X Coefficient(s)	36863
Std Err of Coef.	272

Table 3. Cyanuric acid recoveries from cell culture.

Sample Number	Sample Medium	Conc. of spike (mg/L)	Final conc. (mg/L)	GC vol. inject (μL)	Area (GC)	Percent Recovery (%)	Average Recovery (%)
1/1	Media/cell	4.631	3.789	20	1e+05	100.35	
2/1	Media/cell	4.631	3.789	20	1e+05	100.4	100.38
1/2	Media/cell	4.631	3.789	20	1e+05	100.4	
2/2	Media/cell	4.631	3.789	20	1e+05	99.41	99.91
1/3	Media/cell	4.631	3.789	20	1e+05	99.96	
2/3	Media/cell	4.631	3.789	20	1e+05	99.49	99.72

Linear Regression of Cyanuric Acid Standards:

Standard Number	Concentration (μg/mL)	Retention Time (min)	Area (GC)	Estimated Concentration (μg/mL)
I	0.709	2.955	42553	0.766
II	1.417	2.651	61487	1.433
III	2.362	2.706	85210	2.268
IV	4.724	2.701	154757	4.719
V	7.085	2.693	222663	7.111

Regression Output:

Constant	20822
Std Err of Y Est	1861.1
R^2	0.9995
No. of Observations	5
Degrees of Freedom	3
X Coefficient(s)	28384
Std Err of Coef.	354.98

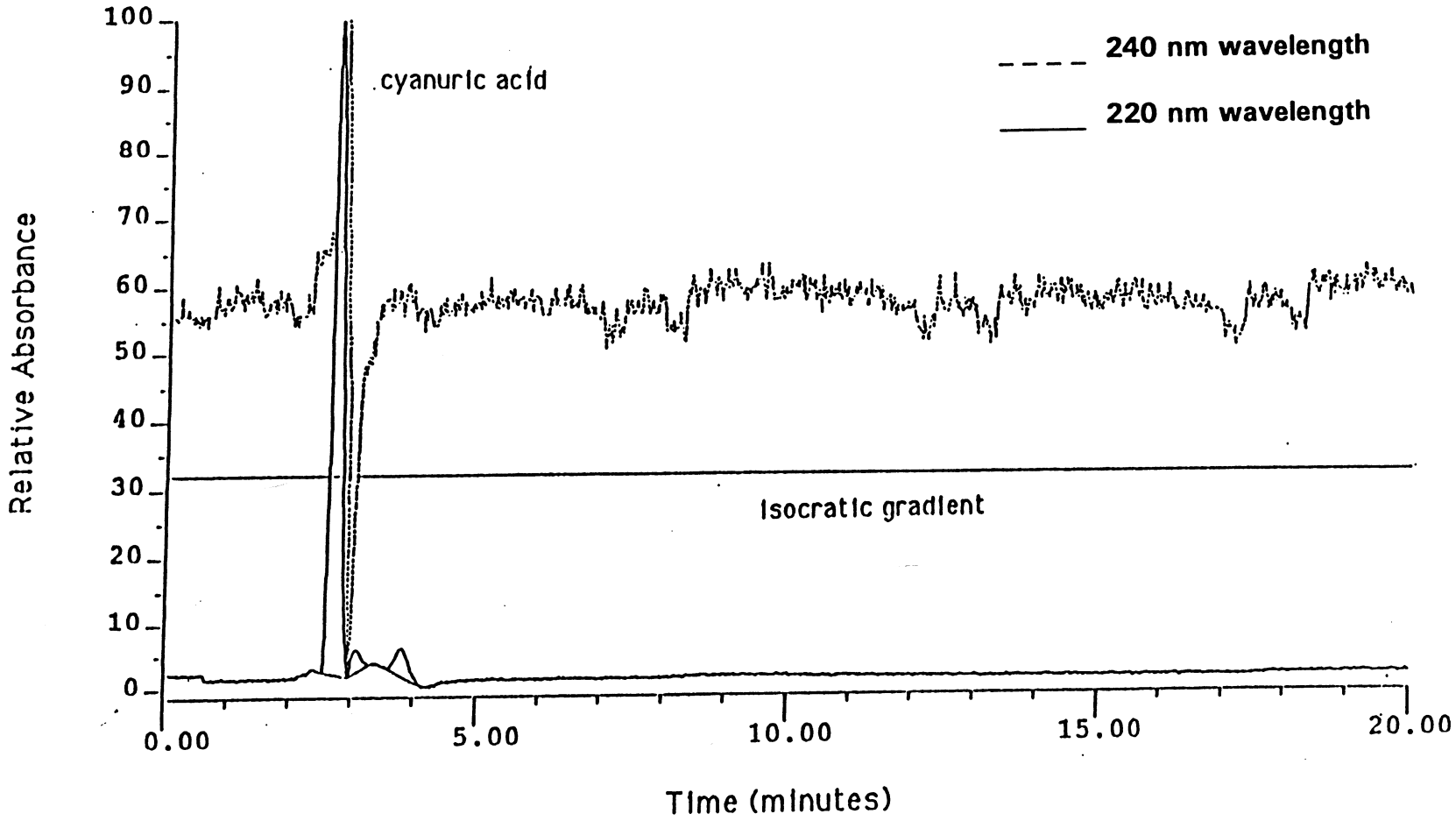


Figure 4. HPLC of cyanuric acid using a mixed mode cation exchange column 7.0 mg/liter standard.

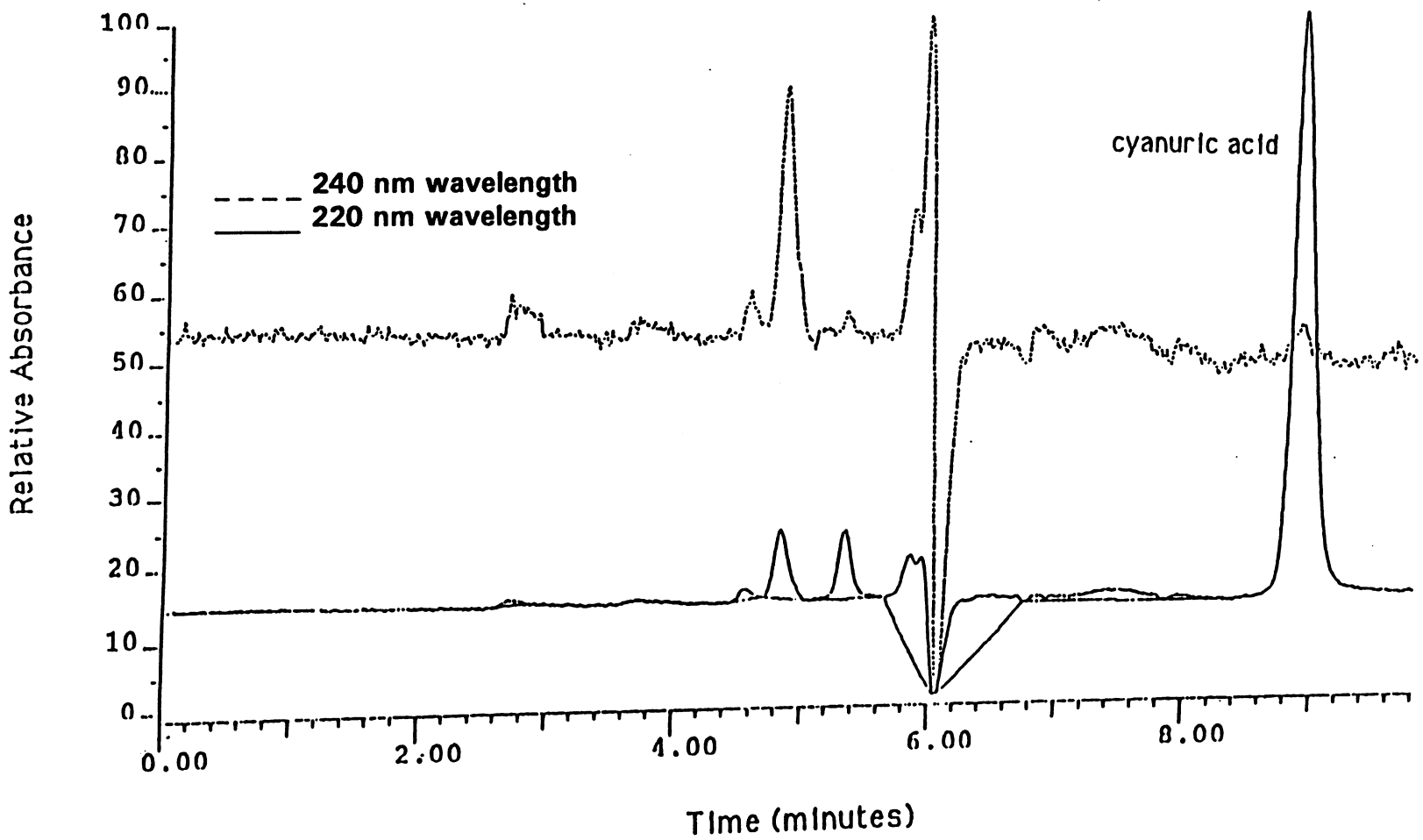


Figure 5. HPLC of cyanuric acid using a C-18 reverse phase (4.7 mg/liter standard).

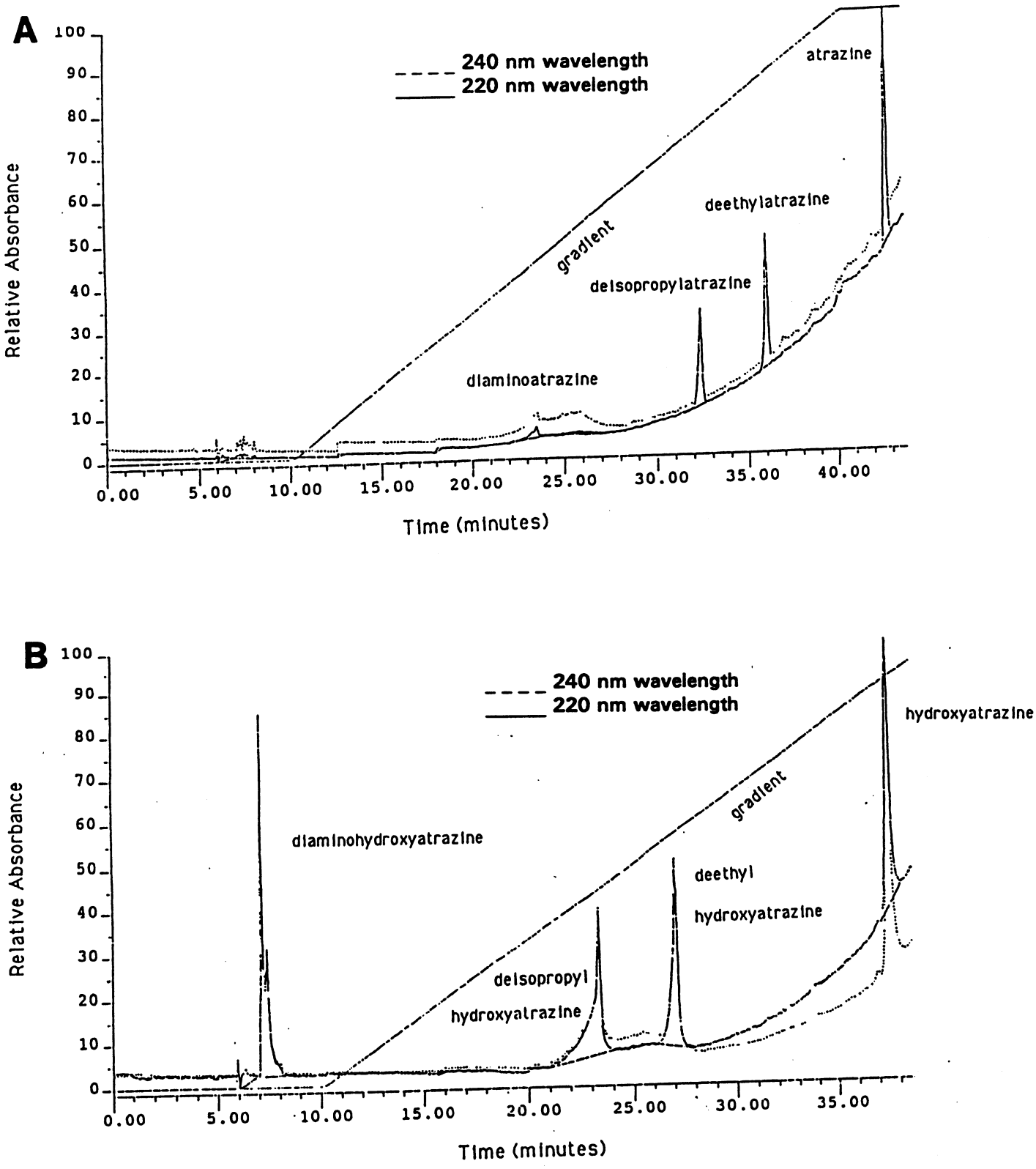


Figure 6. (A) HPLC of chlorinated atrazine metabolites (Beilstein et al., 1981) and (B) HPLC of hydroxylated atrazine metabolites (Beilstein et al., 1981).

Table 4. Results of growth experiments for *Pseudomonas* sp. strain D.

Experiment (substrate)	Protein, %	Growth Yield g protein/N mol	Growth Yield g cells/g glucose	Specific Growth Rate μ hr^{-1}	Nitrogen, %
Nitrogen limited:					
(cyanuric acid)				0.303 ± 0.03	
(ammonium)				0.333 ± 0.01	
Nitrogen limited: (ammonium)	44.61 ± 1.5	45.80 ± 1.5		0.273 ± 0.02	15.22 ± 1.3
Carbon limited: (ammonium)	45.49 ± 4.3		0.344 ± 0.04	0.339 ± 0.02	20.61 ± 2.0

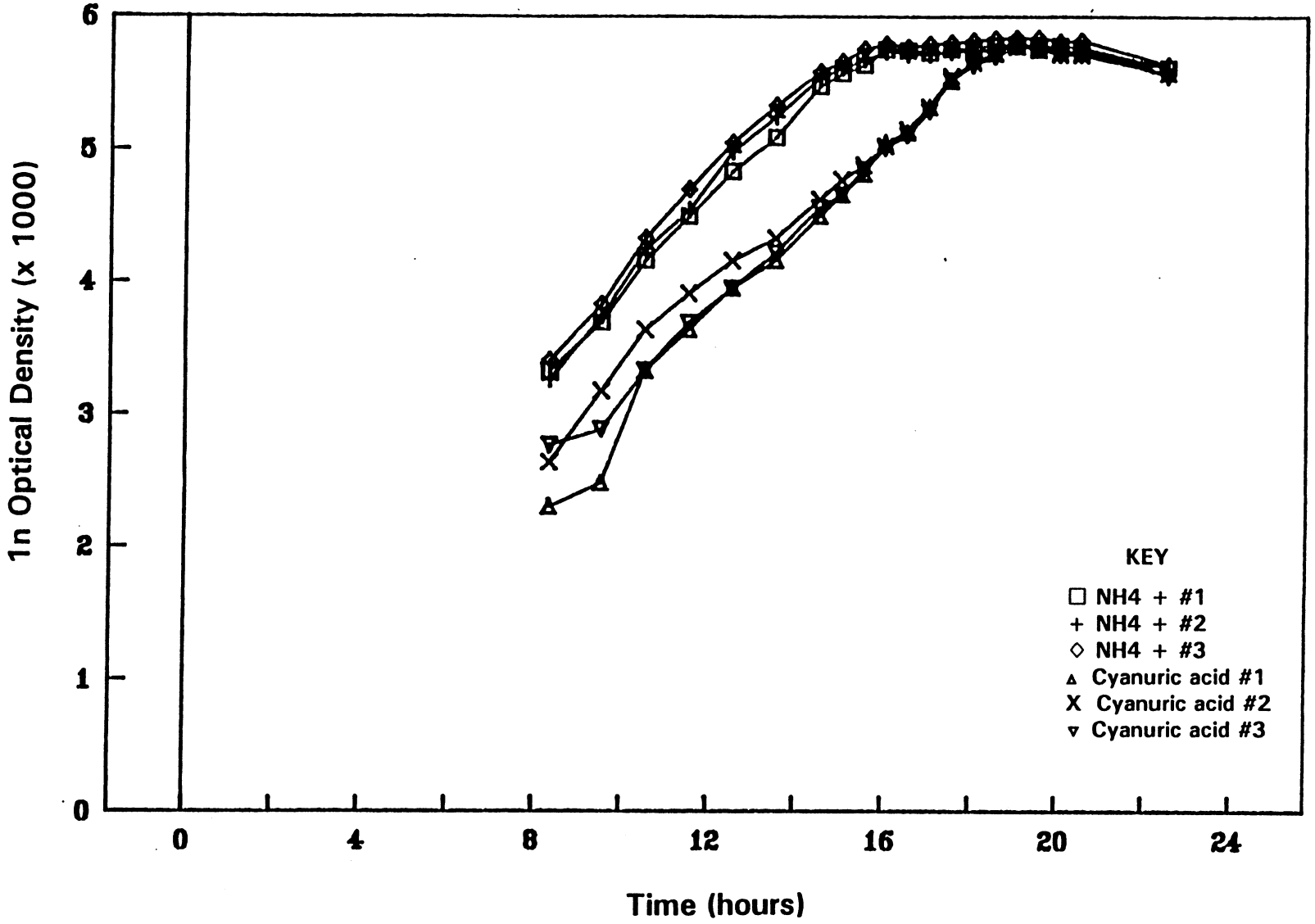


Figure 7. Growth of *Pseudomonas* sp. strain D. utilizing amonium or cyanuric acid under nitrogen limited conditions.

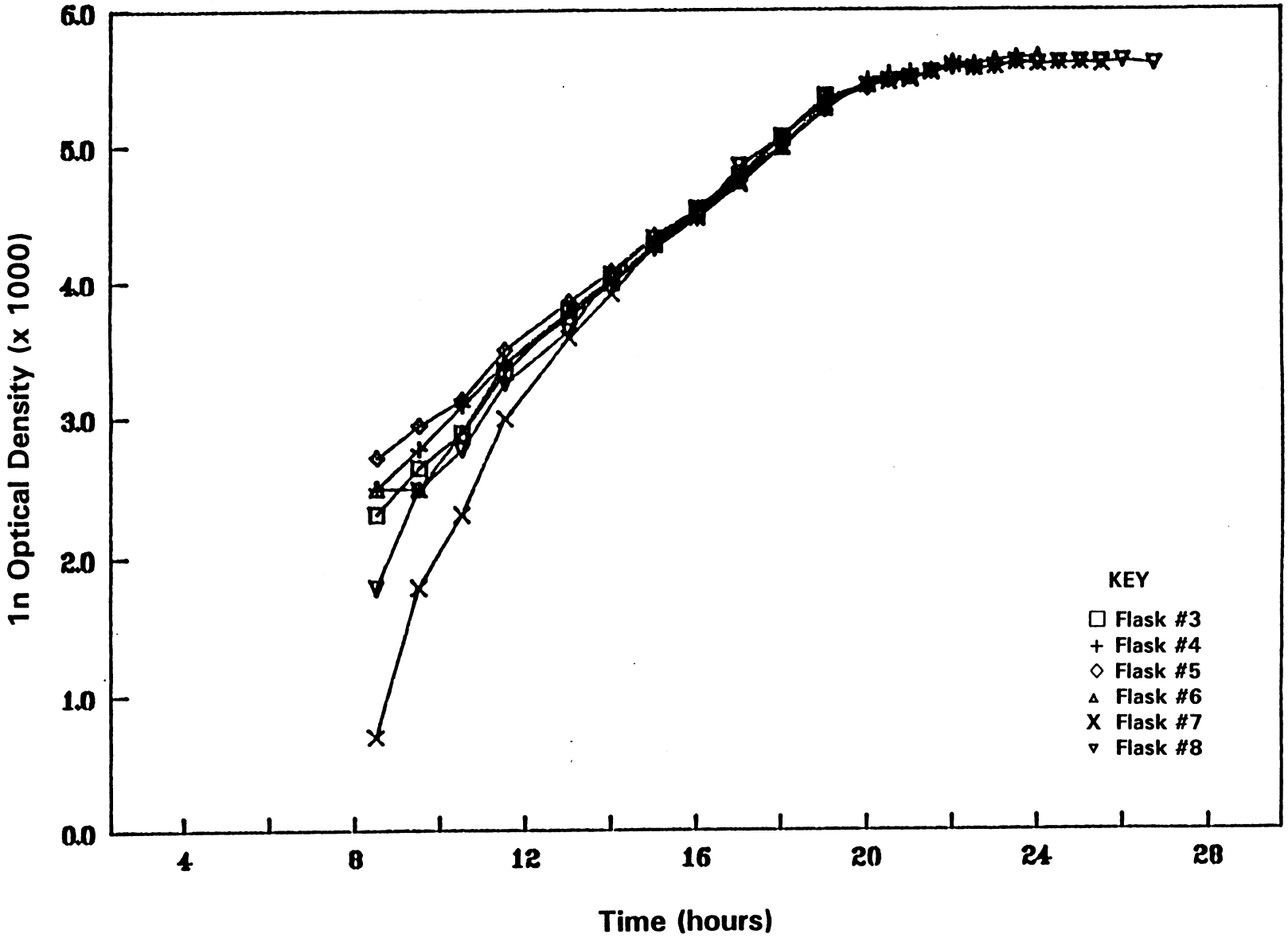


Figure 8. Growth of *Pseudomonas* sp. strain D incubated with limiting nitrogen.

Thus, the value of dry weight is probably low giving a higher percent nitrogen. The specific growth rate, protein content and growth yield all correlate closely with the results of Cook and Hutter (1981), confirming our methodologies. Simulations with our computer models, using a specific growth rate of 0.27 hr^{-1} and a cell composition of 45.8 g protein/mole of cell nitrogen, as inputs, provides added confirmation of the adequacy of the methods. The model simulation predicted 44.3% protein and a growth yield of 46.4 g protein produced/mole of nitrogen consumed, which agree well with experimental values. The model also predicted 13.5% nitrogen which, as expected, is different from experimental values. Thus, the model enables the investigator to evaluate experimental methodologies.

In the third experiment, strain D was grown under carbon limiting conditions with a glucose concentration of 2 m*M* and an ammonium concentration of 2.5 m*M*. From turbidity measurements, an average specific growth rate of 0.34 ± 0.02 hr^{-1} was calculated (Figure 9). The organism had a 45.5 ± 4.3% protein content (which corresponds to a cell composition of 46.7 ± 4.3 g protein/mole of cell nitrogen), a 20.6 ± 2.0% nitrogen content, and average growth yields of 0.34 ± 0.04 g cells produced/gram glucose consumed and 40.6 ± 13.5 g of protein produced/nitrogen mole consumed. Percent nitrogen is much higher than average for a "typical cell" (14%). This may indicate problems with the analysis. The growth yield of 0.34 ± 0.04 g cells produced/gram glucose consumed is much lower than the 0.5 g^{-1} typically found. The low growth yield indicated an analytical problem or that intermediates, not utilized as growth substrates (e.g., acetate), are being produced when the organism utilizes glucose.

Simulations with our computer models, using a specific growth rate of 0.34 hr^{-1}, a cell composition of 46.7 g protein/mole of cell nitrogen, and production of 2 moles of acetate as inputs, gave a growth yield of 0.36 g cells produced/gram glucose consumed. This is close to the actual value of 0.34 ± 0.04 g cells produced/gram glucose strengthening the hypothesis that acetate was being produced. The model simulation predicted a 45.1% protein, also close to the experimental value. The model predicted 13.4% nitrogen, which is much different from the experimental value confirming a problem with methodology.

Growth experiments of strain D utilizing ammonium, and nitrate, cyanuric acid and nitrate, and nitrate alone (under nitrogen limiting conditions at concentrations of 2.5 m*M* total nitrogen) were completed to determine if the organism could utilize nitrate as an assimilatory N source under aerobic conditions. The results show that nitrate is utilized as an assimilatory nitrogen source, but at a much slower rate than cyanuric acid or ammonium. Strain D was unable, however, to utilize nitrate as an electron acceptor under anaerobic conditions. *K. pneumoniae*, on the other hand, was capable of partially reducing nitrate to nitrite, as is expected for enteric organisms. However, nitrite will accumulate and become toxic to the organism and inhibit its growth. Thus, strain D and *K. pneumoniae* would not be useful as model organisms to investigate the degradation of atrazine metabolites under anaerobic conditions.

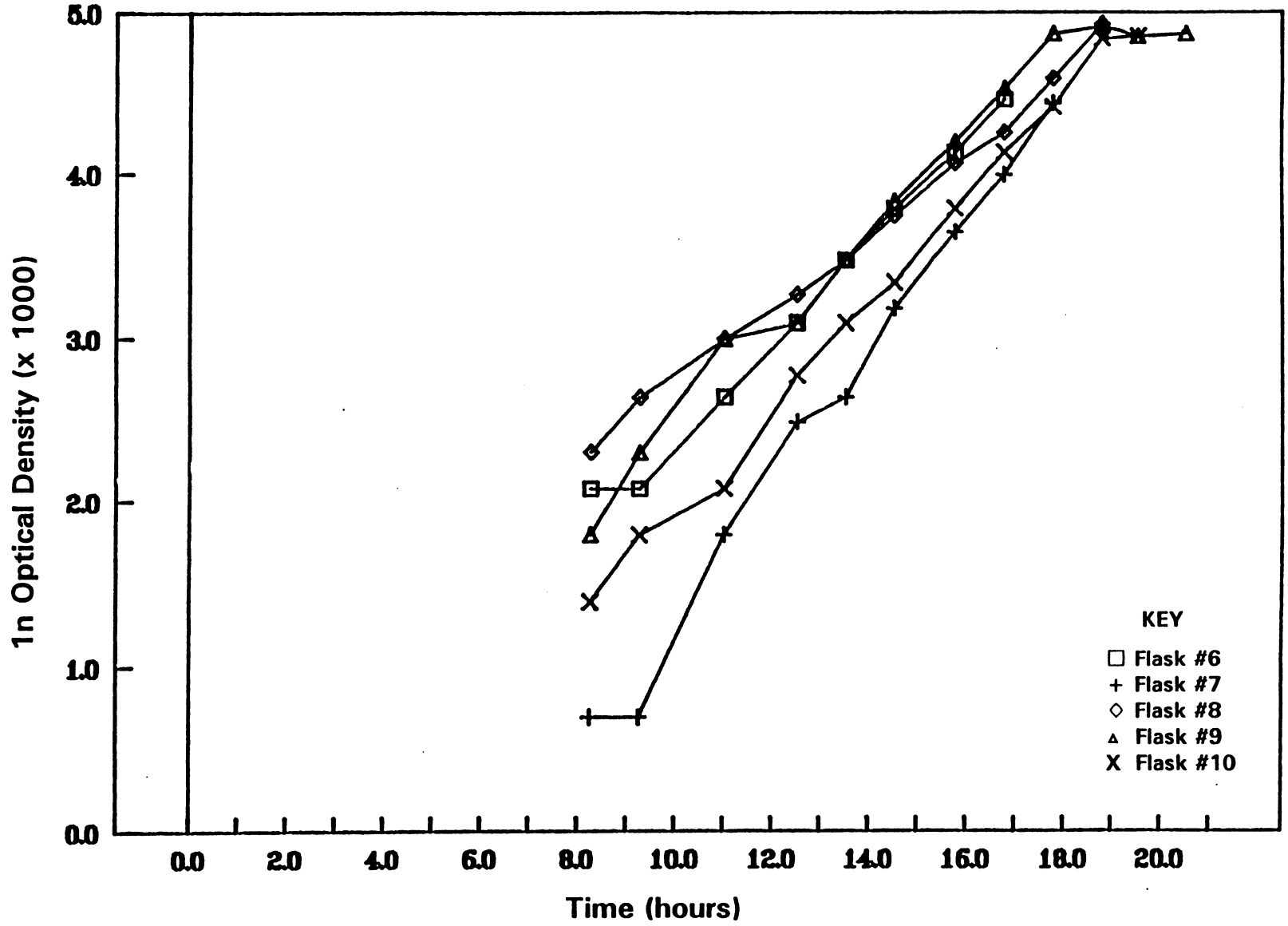


Figure 9. Growth of *Pseudomonas* sp. strain D incubated with limiting carbon.

CONCLUSIONS

The results of the solid phase extraction experiments indicated the need for an internal standard for the gas chromatograph because of the variability between replicate samples. Furthermore, the SPE method is tedious and did not give acceptable recoveries for the hydroxy metabolites of atrazine.

HPLC offers a better alternative for detection of atrazine metabolites because detection of all metabolites including the hydroxy derivatives is possible and the tedious extraction procedure is eliminated. Initial recovery experiments with cyanuric acid indicate this is a highly reliable method. Two HPLC methods have been investigated. The first method used a mixed mode cation exchange column and the chromatogram indicated that cyanuric acid was not retained on the column. The method of Beilstein et al. (1981) showed more promising results. Recent investigations using this method showed excellent separation of seven other atrazine metabolites and the parent compound. The peaks were sharp, but the total run time was 60 min. These and other HPLC methods are currently under investigation to address the run time and peak retention issues. Initial results of strain D growth experiments varied. The data from the nitrogen-limited system, except for a high percent nitrogen, showed very good correlation with the results of our model and Cook and Hutter (1981). However, data from the carbon-limited system did not turn out as expected. The computer model aided in determining problems with the analytical methodology and the build up of intermediates in the carbon-limited experiment.

The utility of the model was verified by these initial experiments. Our experimental methodologies for the determination of atrazine metabolites still needs refining, and the work will focus on the use of HPLC. The biomass methodology also needs some fine-tuning. It is evident that strain D will serve as a useful model organism to further validate the test system prior to beginning enrichment cultures.

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